

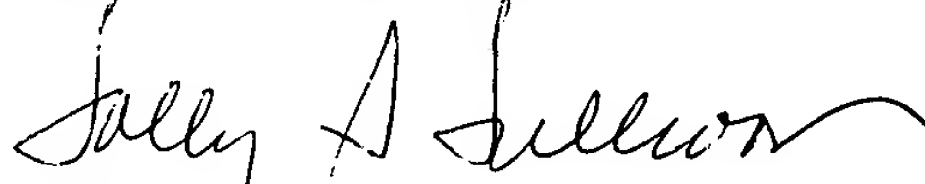
Please delete pages 61 and 62.

REMARKS

This preliminary amendment corrects obvious clerical and typographical errors. It does not represent the addition of new matter to the application. The specification has also been amended to insert references in the text and remove footnotes. Pages 61 and 62 are deleted to remove redundant recitation of references.

It is believed this submission does not require the payment of a fee. If this is incorrect, please deduct the required fee from deposit account 07-1969.

Respectfully submitted,

A handwritten signature in black ink, appearing to read "Sally A. Sullivan", with a stylized flourish at the end.

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Version with markings to show changes made to claims
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1. (Once amended) A substrate for hybridization comprising a plurality of first nucleic [acid] acids alone or in combination with a plurality of one or more oligomers that are not nucleic acids immobilized on at least a portion of the substrate in a medium-high or high immobilization density.
3. (Once amended) The substrate of claim 2 wherein, in an assay, the difference in T_m between
 - [•] (i) a fully-matched complex immobilized to the substrate, the complex comprising the first nucleic acid and the second nucleic acid; and
 - [•] (ii) a mismatched complex immobilized to the substrate, the complex comprising the first nucleic acid and a second nucleic acid having a single nucleotide mismatch;is not decreased compared to the difference in T_m between the complexes in low immobilization density.
7. (Once amended) The substrate of claim 1 wherein the high immobilization density comprises oligomers on the substrate so that the ratio (r_s) of the mean centre-to-centre separation distance of the oligomers to the average length of immobilized oligomers is less than or equal to 1.7.
16. (Once amended) A substrate of [claim1] claim 1 wherein a plurality of first nucleic acids and a plurality of one or more oligomers that are not nucleic acids are immobilized on the substrate.

21. (Once amended) The substrate of claim 1 wherein the interfacial hybridization for fully complementary nucleic acids exhibits enhanced sensitivity to temperature.
22. (Once amended) The substrate of claim [1 wherein] 1 wherein the substrate comprises an optical fiber, an optical wave-guide, a spot on a microarray chip, a microtiter plate well, a metal film for surface plasmon resonance determination, a glass bead, a planar waveguide, a quartz oscillator, a ceramic oscillator, a conductive electrode material, a semi-conductive electrode material, a plastic sample compartment, an optical component, or a pyroelectric material.
32. (Once amended) The method of claim 25 wherein the substrate comprises an optical fiber, an optical wave-guide, a spot on a microarray chip, a microtiter plate well, a metal film for surface plasmon resonance determination, a planar waveguide, a quartz oscillator, a ceramic oscillator, a conductive electrode material, a semi-conductive electrode material, a glass bead, a plastic sample compartment, an optical component, or a pyroelectric material.
33. (Once amended) A method of hybridizing nucleic acids comprising:
 - [•] (i) providing a substrate including a plurality of first nucleic acids or first nucleic acids and oligomers which are not nucleic acids on the substrate, having a medium-high or high immobilization density; and
 - [•] (ii) contacting the substrate with at least one second nucleic acid having a region of contiguous nucleotides that are complementary to all or part of at least one of the first nucleic acids, so that the second nucleic acid hybridizes to the at least one first nucleic acid.

37. (Once amended) The method [any] of claim[s] 33 wherein the second nucleic acid and the at least one first nucleic acid hybridize in a high ionic strength solution.
40. (Once amended) The method of claim 33 wherein the hybridization for fully complementary nucleic acids exhibits enhanced sensitivity to temperature.
40. (Once amended) The method of claim 33 wherein the hybridization for fully complementary nucleic acids exhibits enhanced sensitivity to temperature.
43. (Once amended) The method of claim [33] 35 wherein the first nucleic acids comprise a mixture of nucleic acid sequences.
44. (Once amended) The method of claim 33 wherein the substrate comprises an optical fiber, an optical waveguide, a spot on a microarray chip, a microtiter plate well, a metal film for surface plasmon resonance determination, a planar waveguide, a quartz oscillator, a ceramic oscillator, a conductive electrode material, a semi-conductive electrode material, a glass bead, a plastic sample compartment, an optical component, or a pyroelectric material.
46. (Once amended) The method of claim [33further] 33 further comprising a step of detecting hybridization.
48. (Once amended) A method of detecting the presence of a genetic target in a test sample, comprising:
 - [•] (i) providing a substrate including a plurality of genetic marker nucleic acids immobilized to the substrate, alone or in combination with one or more oligomers at a medium-high or high immobilization density;
 - [•] (ii) contacting the substrate with a test sample comprising a mixture of nucleic acids so that a second nucleic acid having a region of contiguous nucleotides that are

complementary to all or part of at least one of the genetic marker nucleic acids hybridizes to at least one first nucleic acid; and

- [•] (iii) detecting hybridization of the genetic marker to the second nucleic acid, wherein hybridization is indicative of the presence of a genetic target in the sample.

50. (Once amended) The method of claim 48 wherein, in an assay, the difference in T_m between

- [•] (i) a fully-matched complex immobilized to a substrate, the complex comprising the first nucleic acid and the second nucleic acid; and

- [•] (ii) a mismatch complex immobilized to a substrate, the complex comprising the first nucleic acid and a second nucleic acid having a single nucleotide mismatch

is increased or maintained relative to the difference in T_m between the complexes in low immobilization density.

55. (Once amended) The method of claim 48 wherein the hybridization for fully complementary nucleic acids exhibits enhanced sensitivity to [temperature] temperature.

58. (Once amended) The method of claim [48] 50 wherein the first nucleic acids comprise a mixture of nucleic acid sequences.

59. (Once amended) The method of claim 48 wherein the substrate comprises an optical fiber, an optical waveguide, a spot on a microarray chip, a microtiter plate well, a metal film for surface plasmon resonance determination a planar waveguide, a quartz oscillator, a ceramic oscillator, a conductive electrode material, a semi-conductive electrode material, a glass bead, a plastic sample compartment, an optical component, or a pyroelectric material.

62. (Once amended) The method of claim 48 wherein the genetic marker nucleic acids are derived by a nucleic acid amplification method.
63. (Once amended) The method of claim 48 wherein the genetic target comprises an environmental marker nucleic acid, a food marker nucleic acid or a biowarfare agent nucleic acid[and wherein hybridization is indicative of the presence of the genetic target in the sample].
67. (Once amended) The method of claim [48wherein] 48 wherein the nucleic acids to be tested comprise an indicator agent.
- 75.____(Once amended) A method for identifying or isolating a target nucleic acid from a mixture containing nucleic acids which comprises the steps of:
- (i)____providing a substrate of claim 1 wherein the first nucleic acids comprise a sequence that is complementary at least in part to the target nucleic acid; and
- (ii)____contacting the substrate with the mixture containing nucleic acids such that any target nucleic acid present in the mixture can hybridize to the first nucleic acids on the substrate.
- 76.____(Once amended) The method of claim 75 wherein the step of contacting the substrate with the mixture is performed at high ionic strength.
- 77.____(Once amended) The method of claim 75 wherein the mixture containing nucleic acids can contain nucleic acids that differ from the target nucleic acid by a single base change.

Page 1, third full paragraph:

The immobilization of biomolecules to solid surfaces is widely used in the preparation of analytical sensors. Applications include immunosensor techniques [[1,2,3]] (R. Blonder, E. Katz, Y. Cohen, N. Itzhak, A. Riklin, I. Willner, Anal. Chem., v. 68, p. 3151, 1996; R. Granzow and R. Reed, Biotechnology, v. 10, p. 390, 1992; B. König and M. Grätzel, Anal. Chim. Acta, v. 309, p. 19, 1995), which tend to rely on protein binding as the means of molecular “recognition”, as well as those which make use of nucleic acid hybridization [[4,5,6,7,8,9]] (K.M. Millan, A. Saraullo, and S.M. Mikkelsen, Anal. Chem., v. 66, p. 2943, 1994; J. Wang, S. Bollo, J.L. Lopez Paz, E. Sahlin and B. Mukherjee, Anal. Chem., v. 71, p. 1910, 1999; H. Su, K.M.R. Kallury, M. Thompson and A. Roach, Anal. Chem., v. 66, p. 769, 1994; F. Caruso, E. Rodda, D.N. Furlong, K. Niikura, and Y. Okahata, Anal. Chem., v. 69, p. 2043, 1997; A.P. Abel, M.G. Weller, G.L. Duveneck, M. Ehrat and H.M. Widmer, Anal. Chem., v. 68, p. 2905, 1996; P.A.E. Piunno, U.J. Krull, R.H.E. Hudson, M.J. Damha and H. Cohen, Anal. Chem., v. 67, p. 2635, 1995) as the basis for selective recognition. The use of immobilized nucleic acids to provide for selective binding interactions is attractive since the selectivity of nucleic acid binding interactions can be quite high and the advent of polymerase chain reaction and solid phase nucleic acid synthesis has allowed for relatively simple nucleic acid preparation and immobilization.

Page 1, fourth full paragraph:

The utility of immobilized selective molecular recognition elements is dependent upon the retention of selective binding capacity after the immobilization process is complete. The binding capacity is dependent upon the structure of the immobilized molecules in their local environments, which can be significantly different from those experienced in bulk solution. The density of immobilization of single-stranded DNA (ssDNA) onto the surface of a solid substrate affects the charge density at the surface, and the extent to which the immobilized oligomers interact with the surface of the solid substrate and with neighbouring nucleic acid oligomers. The density of immobilization thus affects the extent of hybridization, as well as the orientation of the immobilized ssDNA, and therefore affects the kinetics of hybridization [[10]] (H. Su, P. Williams and M. Thompson, Anal. Chem., v. 67 p. 1010, 1995). Clearly, the control of

selectivity of binding and the dynamic range that can be achieved by control of the concentration of oligonucleotide sequences at an interface is complex.

Paragraph bridging pages 1 and 2:

The binding capacity of immobilized oligonucleotides is dependent in part upon the structure and orientation of the oligonucleotides in the interfacial environment, which is dictated at least in part by chemical nature of the solid substrate. Control and elucidation of the orientation and packing structure of nucleic acids immobilized on gold and polystyrene surfaces has been attempted [[11,12,13]] (T.M. Herne and M.J. Tarlov, J. Am. Chem. Soc., v. 119, p. 8916, 1997; R. Levicky, T.M. Herne, M.J. Tarlov, and S.K. Satija, J. Am. Chem. Soc., v. 120, p. 9787, 1998; M.-T. Charreyre, O. Tcherkassaya, *et al.* Langmuir, v. 13, p. 3103, 1997). It was suggested that the alignment of immobilized oligonucleotides with respect to the substrate surface can be controlled by selection of oligonucleotide immobilization density, as well as through control of the chemical environment at the surface. For example, Tarlov *et al.* [[11]] (T.M. Herne and M.J. Tarlov, J. Am. Chem. Soc., v. 119, p. 8916, 1997) reported that adsorptive interactions of oligonucleotides immobilized by sulfur-gold interactions on a gold surface were reduced by blocking unreacted surface sites with mercaptohexanol. The reduction in oligonucleotide adsorption to gold resulted in extension of the immobilized oligonucleotides away from the substrate surface. The extent of hybridization was found to be affected by the packing density of immobilized oligonucleotides, with hybridization being inhibited at higher packing densities where steric hindrance and electrostatic repulsion were thought to reduce the stability of hybrids that could form. Alternatively, Fotin *et al.* [[14]] (A.V. Fotin, A.L. Drobyshev, D.Y. Proudnikov, A.N. Perov, A.D. Mirzabekov, Nuc. Ac. Res., v. 26, p. 1515, 1998) reported a method for large-scale parallel thermodynamic analysis of oligonucleotide hybridization using oligonucleotides immobilized in an array of polyacrylamide gel pads, each of dimension $100 \times 100 \times 20 \mu\text{m}$. This method of immobilization was claimed to be well-suited for large scale thermodynamic analysis of oligonucleotide hybridization because the local environment experienced by the immobilized oligonucleotides afforded by the polyacrylamide gel more closely resembled that of a homogeneous liquid phase than that of the heterogeneous solid-liquid interface obtained when DNA is immobilized onto gold, silica, or polystyrene. Consequently, the method was presented as a means to estimate thermodynamic properties of

oligonucleotide hybrids in solution based on the properties observed in experiments done within the gel-pad environment.

Paragraph bridging pages 2 and 3:

The binding capacity of immobilized nucleic acids is also dependent upon the extent to which neighbouring oligonucleotides can interact with each other. Shchepinov *et al.* [[15]] (M.S. Shchepinov, S.C. Case-Green, and E.M. Southern, Nuc. Ac. Res., v. 25, p. 1155, 1997) reported on the effects of the length of the linker molecule separating the immobilized oligonucleotide from the solid substrate surface on the extent of hybridization. They reported the observation of an optimal linker length of approximately 40 atoms, beyond which reductions in hybridization efficiency were attributed to increased interactions between neighbouring oligonucleotides that imparted steric hindrance to hybridization. It has also been suggested that the density of immobilization of oligonucleotides on polystyrene latex particles effects the orientation of the immobilized strands relative to the surface. Winnik *et al.* [[14]] (A.V. Fotin, A.L. Drobyshev, D.Y. Proudnikov, A.N. Perov, A.D. Mirzabekov, Nuc. Ac. Res., v. 26, p. 1515, 1998) used fluorescence resonance energy transfer (FRET) to examine the proximity of fluorescein-labelled oligonucleotides (donor) to immobilized tetramethylrhodamine moieties (acceptor), and thereby give a relative measure of immobilized oligonucleotide conformation relative to the substrate surface under a variety of experimental conditions. In addition to reporting that solution conditions such as pH and ionic strength affect the conformation of immobilized oligonucleotides, they reported that increasing the density of immobilized oligonucleotides also reduced the extent of energy transfer between the fluorescein and tetramethylrhodamine moieties, suggesting that as oligonucleotide packing density increased, the immobilized strands extended further away from the substrate surface due to electrostatic repulsion between neighbouring polyanionic strands.

Page 3, first full paragraph:

The development of microarray technologies has stemmed from the desire to examine very large numbers of nucleic acid probe sequences simultaneously, in an effort to obtain information about genetic mutations, gene expression or nucleic acid sequences. Microarray technologies are intimately connected with the Human Genome Project, which has development of rapid methods of nucleic acid sequencing and genome analysis as key objectives [[16]] (E.

Marshall, Science, v. 268, p. 1270 1995). Genome mapping and elucidation of sequence-function relationships will provide a wealth of knowledge about all stages of human development and aging, as well as, the onset of and predisposition to disease [[17]] (M. Schena, D. Shalow. R. Heller, A. Chai, P.O. Brown, R.W. Davis, Proc. Nat'l. Acad. Sci. USA, v.93, p.10614, 1996).

Paragraph bridging pages 3 and 4:

Oligonucleotide arrays have been developed as a hybridization “template” where a target sequence can be examined for its ability to hybridize to large numbers of different immobilized oligonucleotide sequences. These systems have been the focus of much research and have been reviewed [[18,19]] (“Recent Advances in Environmental Chemical Sensors and Biosensors”, ACS Symposium Series, in press; M. Thompson and L. M. Furtado, Analyst, v. 124, p. 1133, 1999). One such system has been developed at Affymetrix, Inc. [[20]] (Affymetrix, Inc., 3380 Central Expressway, Santa Clara, California, USA) that makes use of photolithographic techniques to direct spatially addressed synthesis of polynucleotides [[21]] (S.P.A. Fodor, J.L. Read, M.C. Pirrung, L. Stryer, A. Tsai Lu, D. Solas, Science, v.251, p.767, 1991). Arrays are synthesized on solid glass supports that have been coated with amino-terminated linkers to which photolabile nitroveratryloxycarbonyl (NVOC) groups have been added. Photo-deprotection of selected areas is achieved by illuminating those target areas through a photolithographic mask. Subsequent exposure of the entire chip to amino acid or nucleotide reagents results in reaction only at the selectively deprotected sites. Thus, site-specific synthesis is achieved through repetition of these steps and use of the appropriate photolithographic masks. Hybridization of these probe sequences with fluorescently-labelled target [polynucleotidescan] polynucleotides can then be done and the array can be scanned by means of scanning fluorescence microscopy. The fluorescence patterns are then analyzed by an algorithm that determines the extent of mismatch content, identifies polymorphisms and can provide some general sequencing information [[22]] (M. Chee, R. Yang, E. Hubbell, A. Berno, S.C. Huang, D. Stern, J. Winkler, D.J. Lockhart, M.S. Morris, S.P.A. Fodor, Science, v. 274, p. 610, 1996). Selectivity is afforded in this system by low stringency washes to rinse away non-selectively adsorbed materials. Subsequent analysis of relative binding signals from array elements determines where base-pair mismatches may exist. This method then relies on conventional chemical methods to maximize

stringency, and automated pattern recognition processing is used to discriminate between fully complementary and partially complementary binding.

Paragraph bridging pages 4 and 5:

Another oligonucleotide array system has been developed by Nanogen Inc. [[23]] ((i) Nanogen, Inc., 10398 Pacific Center Court, San Diego, California, USA, 27, (ii) R.G. Sosnowski, E. Tu, W.F. Butler, J.P. O'Connell, M.J. Heller, Proc. Natl. Acad. Sci., v. 94, pp. 1119-1123, 1997). An array of platinum microelectrodes was fabricated on silicon wafers using photolithography. One example of such an array device consisted of 25 microelectrodes, 80 μm in diameter, and four microelectrodes, 160 μm in diameter occupying outer corner positions of the array. Each electrode was covered with an agarose permeation layer that permitted ion transport to and from the electrode surface while serving as a site for attachment of probe oligonucleotides. The permeation layer also served as a "spacer" layer that acted to sufficiently separate the probe oligonucleotides from the electrode surfaces to protect the DNA from damaging redox reaction sites. Each electrode in the array was independently connected to an external power source. A continuously adjustable potential or current could be directed to each electrode via computer-controlled switching. This allowed each electrode to be maintained at a positive, negative or neutral bias with respect to the power supply. In one example, immobilization of probe DNA was achieved by incorporating streptavidin into the agarose permeation layer and directing biotinylated oligonucleotides to the layer by applying a positive potential at the target electrode sites. The extent of immobilization using positive, negative and neutral biases was examined by using fluorescently labelled oligonucleotides in the immobilization. It was observed that significant immobilization occurred only at those sites that were at a positive applied potential. This immobilization was also observed to be irreversible by switching the potential of the electrode and applying a strong negative potential. Hybridization of labelled target DNA was then carried out using electric field control as described above. It was found that hybridization to complementary DNA immobilized at electrodes with a positive applied potential occurred 25 times faster than hybridization at neutral electrodes. Reversal of the electric field was then used to examine the ability of the system to discriminate between hybrids of complete complementarity and those that contained single base-pair mismatches. It was observed that electrodes where hybrids were completely complementary retained 70% of the

original fluorescent signal, whereas electrodes where hybrids contained single base-pair mismatches retained only 13% of the original fluorescent signal (i.e., a selectivity ratio of only about 5.4). This ability to discriminate between fully complementary hybrids and those containing single base-pair mismatches was observed with hybrids of different length and G-C content, and was found to occur quite rapidly, with full signal achieved in 15 seconds or less. Overall, this system is significant since it shows that controlling the electrochemical environment of the hybrids affects the selectivity of hybridization in an assay.

Page 5, third full paragraph:

The invention relates to methods for increasing the selectivity of hybridization of probe nucleic acids [immobilized] immobilised on substrate surfaces to other nucleic acids. The methods of this invention can be used to increase selectivity in nucleic acid diagnostic devices, such as biosensors and microarrays, which detect the presence of nucleic acid in a test sample through detection of hybridization between the [immobilized] immobilised probe nucleic acid and nucleic acids in a test sample. The invention provides increased selectivity through control of the substrate surface chemistry and in particular, through control of the density of nucleic acids and other oligomers immobilised on a surface. The invention provides improved signal to noise in hybridization assays via enhanced differences in signal magnitude generated for fully matched target nucleic acid as opposed to partially matched target nucleic acid prior to signal processing. This makes the task of signal processing less onerous, time consuming and complex. Furthermore, control of the substrate surface chemistry can be used to adjust the effective duplex melting temperature (T_m) so that combinations or arrays of immobilised nucleic acid films (a layer of immobilized [oligomers]in] oligomers) in a system can be made to be of similar T_m , regardless of immobilized nucleotide length and sequence. This will allow for simultaneous analysis of many interfacial hybridisations, facilitating enhanced high throughput screening capacity. The properties of immobilized nucleic acids described in this invention are applicable to many different devices using various types of nucleic acid immobilization strategies that will be apparent to one of ordinary skill in the art.

Paragraph bridging pages 5 and 6:

Furthermore, control of the substrate surface chemistry can be used to adjust the effective duplex melting temperature (T_m) so that combinations or arrays of immobilised nucleic acid films (a layer of immobilized oligomers) in a system can be made to be of similar T_m , regardless of immobilized nucleotide length and sequence. This will allow for simultaneous analysis of many interfacial hybridisations, facilitating enhanced high throughput screening capacity. The properties of immobilized nucleic acids described in this invention are applicable to many different devices using various types of nucleic acid immobilization strategies that will be apparent to one of ordinary skill in the art.

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Page 15, first full paragraph:

Other specific embodiments of the invention relate to a substrate for hybridization, comprising a plurality of first nucleic acids immobilized on the substrate and a plurality of oligomers other than nucleic acids immobilized on the substrate. It will be apparent to a skilled artisan how to adapt the teachings in this application for use with oligomers other than nucleic acids. The oligomers other than nucleic acids can be similar or different in length from the nucleic acids with which they are co-immobilized. Oligomers other than nucleic acids that can be used in preparation of these substrates can be linear or branched in [strucutre] structure and can include [with out] without limitations polyethers which may be linear or branched. The relative amounts of nucleic acids to oligomers that are not nucleic acids that are immobilized on a substrate can vary widely. The number ratio (or molar ratio) of nucleic acids to other oligomers can, for example, vary from 1:1 up to 1:1000. In specific embodiments this ratio can be 1:10, 1:20, 1: 50, 1:100 or 1:500.

Paragraph bridging pages 16 and 17:

In a preferred embodiment of the hybridization and assay methods herein employing a substrate a medium-high or high immobilization density of oligomers including a plurality of first nucleic acids, a second nucleic acid having a region of contiguous nucleotides that is complementary to all or part of at least one of the first nucleic acids that will selectively hybridize to the at least one first nucleic acid. Also, preferably, in an *in-vitro* assay, the difference in T_m between

Page 20, first full paragraph:

Control of oligonucleotide immobilization density and organisation in devices that make use of covalently immobilized nucleic acids may be achieved by control of the number of available reactive sites on a substrate onto which the oligonucleotides and any oligomers that are not nucleic acids will be immobilized. Since it is desirable to immobilize the nucleic acids to the substrate surface *via* appropriate linker molecules (e.g., polyether or hydrocarbon chains (T.M. Herne and M.J. Tarlov, J. Am. Chem. Soc., v. 119, p. 8916, 1997; U. Maskos and E.M. Southern, Nuc. Acids Res., v. 20, p. 1679, 1992.)), control of immobilization density can be afforded through control of the immobilization density of linker molecules, however other methods can be employed to control immobilization density. An exemplary method for controlling immobilization density control is by control of the density of polyether linker moieties on a substrate Preferred polyether type linker molecules are greater than about 20 and less than about 40 atoms in length [M.S. Shchepinov, S.C. Case-Green, and E.M. Southern, Nucleic Acids Research, v. 25, 1997, p. 1155]. Linker structures can also include dendritic forms of poly(ethylene oxide) chains, such as have found application in the preparation of nucleic acid microarray substrates [M. Beier and J.D. Hoheisel, Nucleic Acids Research, v. 27, 1999, pp. 1970-1977]. Linkers can also be hydrocarbon based and more [preferable] preferably contain electronegative moieties within them, such as oxygen, to minimize associative interactions [S.L. Beaucage and R.P. Iyer, *Tetrahedron*, v. 48, 1992, pp. 2223-2311]. Linkers are preferably longer than 18 atoms [S.L. Beaucage and R.P. Iyer, *Tetrahedron*, v. 48, 1992, pp. 2223-2311]. References in this application to the nucleic acid:linker refer to a situation where a

nucleic acid is tethered to a substrate by a linker as well as the situation where there is no linker and the nucleic acid is immobilized directly on the substrate.

Paragraph bridging pages 20 and 21:

The selectivity and sensitivity of hybridization assays exemplified herein were performed using nucleic acid biosensors with controlled immobilization densities of oligonucleotides alone or in combination with oligomers that are not nucleic acids. The measurement technique employed was based on total internal reflection fluorescence (TIRF), which has been described in detail [[28]] (P.A.E. Piunno, J.H. Watterson, C.C. Wust, and U.J. Krull, *Anal. Chim. Acta* v. 400, p. 73, 1999). Thermodynamic selectivity and the thermodynamic stability of hybrids formed in an interfacial environment were examined by use of thermal denaturation profiles collected using this instrument. These profiles provided the necessary information to determine thermodynamic parameters such as the thermal denaturation temperature (T_m , or temperature at which 50% of all duplexes formed are denatured), van't Hoff enthalpy change (ΔH_{VH}) and standard enthalpy change (ΔH°) of the denaturation transition.

Page 22, lines 21-29:

In another example, the plastic surface can be aminated by gas plasma reaction chemistry in a nitrogen rich environment. Phosphoramidite synthons of the linker molecules could then be immobilised in controlled fashion through control of reaction conditions (e.g., reaction time, reactant concentration, temperature, and/or choice of solvent conditions) which in turn would provide template sites for oligonucleotide attachment. This can be done, for example, on either hydroxylated or aminated plastic substrates. The general chemistry for attachment of phosphoramidite synthons of the linker molecule to a hydroxylated or aminated surface would preferably follow the well-established solid-phase [β -cyanoethylphosphoramidite] β -cyanoethylphosphoramidite chemistry as used for nucleic acid synthesis in DNA synthesizers.

Page 36, lines 8-24:

4.1.1: Preparation of (3,5-Dimethoxy-phenyl)-(2-phenyl-[1,3]dithian-2-yl)-methanol
[¹](Stowell, Michael H.B. *et al.* *Tetrahedron Letters*, 1996, vol. 37, No. 3, pp.307-310).

A solution of 2-phenyl-1,3-dithiane [²](White crystalline solid with strong odour. Handle in glove box.) (5.0 g, 0.0255 mol) in 85 ml of anhydrous tetrahydrofuran [³] (Moisture determination done by Coulometric Karl Fischer analysis: 55 ppm H₂O) (THF) was cooled to 0 °C [⁴](Exact temperature is not required. Ice-water bath is sufficient.) and 1.05 equivalents of *n*BuLi (10.7 ml, 2.5 M solution in hexane) [⁵] (Pale yellow liquid. **FLAMMABLE** upon exposure to moisture. Store at 0-5°C. Prior to use allow bottle to warm to room temperature. **DO NOT** remove sur-seal. All transfers should be done by syringe under an inert atmosphere) was added dropwise *via* syringe with rapid stirring [⁶](Accomplished by use of a magnetic stir bar and stir plate. Solution became yellow in colour.), under an inert atmosphere of nitrogen. This solution was allowed to stir for 30 minutes at 0 °C and then 1.0 equivalents of 3,5-dimethoxybenzaldehyde [⁷] (White crystalline solid. Handle in glove box.) (4.23 g, 0.0255 mol), dissolved in a minimal amount of anhydrous tetrahydrofuran [³] (Moisture determination done by Coulometric Karl Fischer analysis: 55 ppm H₂O), was added dropwise over a period of 30 min. The solution was allowed to warm to room temperature [⁸] (Requires approx. 2-2.5 hours.) and then stirred for an additional hour. The reaction is quenched by the addition of aqueous NH₄Cl. Tetrahydrofuran was removed *in vacuo* and the resultant slurry extracted with dichloromethane [⁹] (Distilled, not anhydrous, dichloromethane was sufficient.) (100 ml). The organic phase was washed with 3 × 50 ml of distilled water [¹⁰] (Extraction of the two phases was done using a separatory funnel. The organic phase (composed mostly of dichloromethane) was recovered as the heavier phase owing to the greater density of the organic solvent to that of water.), brine (1 × 50 ml), dried (Na₂SO₄) [¹¹] ([Anhydrous] Anhydrous sodium sulphate was used to remove water (2 or 3 spatula scoops)), filtered [¹²](Gravity filtration using fluted filter paper.) and concentrated *in vacuo* to yield crude material as a pale yellow oil. Column chromatography [¹³] (Flash chromatography is used. This involved the application of air pressure onto the solvent above the chromatography media in order to expedite the elution process.) (silica gel, Hexane:Dichloromethane / 7:3, R_f = 0.0, followed by Dichloromethane, R_f = 0.1) [¹⁴] (Once all other impurities (which have higher R_f's) are eluted, the eluent is switched to dichloromethane (100%) to speed up the recovery of the desired product.) yielded 7.4 g (80%) of pure product [¹⁵] (Hygroscopic white foam.). δ_H(200 MHz; CDCl₃) 7.77-7.72 (2 H, m, aryl), 7.34-7.28 (3 H, m, aryl), 6.31 (1 H, t, *J* 2.2, aryl), 6.00 (2 H, d, *J* 2.2, aryl), 4.96 (1 H, bs, CH-OH), 3.59 (6 H, bs,

CH₃O), 2.99 (1 H, bs, OH), 2.77-2.68 (4 H, m, (S-CH₂)) and 2.03-1.92 (2 H, m, CH₂); *m/z* (EI) 362 (M⁺, 5%), 287 (25), 256 (75), 195 (100).

Page 37, lines 16-27:

4.1.2: Preparation of 2-(3,5-Dimethoxy-phenyl)-2-hydroxy-1-phenyl-ethanone (DMB-OH) [^{1a,b}] (Stork, Gilbert; Zhao, Kang, Tetrahedron Letters, 1989, Vol. 30, No. 3, pp.287-290; Photolysis occurs in standard laboratory light and product must be kept in complete darkness. Reaction must be performed in the dark or under red light (> 630nm wavelength)) Bis(trifluoroacetoxy)iodobenzene [²] (Pale yellow solid. Handle in glove box. Keep under an inert atmosphere until needed.) (3.4 g, 0.0084 mol) was added at room temperature to a stirred solution of the dithiane benzoin adduct (2.45 g, 0.0067 mol) dissolved in 15 ml of acetonitrile:water / 9:1. [³] (HPLC grade acetonitrile and milli-Q water is used.) The reaction mixture was then stirred for 2.5 hours [⁴] (Solution turned a pale orange-yellow). Saturated aqueous sodium bicarbonate (75 ml) was added followed by extraction of the mixture into dichloromethane [⁵](Distilled, not anhydrous, dichloromethane is sufficient. Extraction of the two phases was performed using a separatory funnel. Dichloromethane is the bottom phase as its density is greater.) (75 ml). The aqueous layer was further washed with dichloromethane (3 × 25 ml). The organic layer was then dried (Na₂SO₄) [⁶] ([Anhydrous] Anhydrous sodium sulfate is used to remove any trace of water (2 or 3 spatula scoops)), filtered [⁷] (Gravity filtration using fluted filter paper.) and concentrated in vacuo to yield crude material as a pale yellow solid. Column chromatography [⁸] (Flash chromatography is used. This involves the application of air pressure onto the solvent above the chromatography media in order to expedite the elution process.) (silica gel, dichloromethane, R_f = 0.15) [⁹] (Once all other impurities (which have higher R_f's) are eluted, the eluent is switched to dichloromethane:ether / 1:1 to speed up the recovery of the desired product.) yielded 1.26 g (69%) of pure product [¹⁰] (Pale yellow solid.). δ_H(200 MHz; CDCl₃) 7.97-7.92 (2 H, m, aryl), 7.56-7.38 (3 H, m, aryl), 6.49 (2 H, d, J 2.2, aryl), 6.37 (1 H, t, J 2.2, aryl), 5.87 (1 H, d, J 6.2, CH-OH), 4.54 (1 H, d, J 6.2, OH) and (6 H, s, CH₃O); *m/z* (EI) 272 (M⁺, 34%), 167 (100), 139 (69), 105 (54), 77 (44).

4.1.3: Preparation of Carbonic acid 1-(3,5-dimethoxy-phenyl)-2-oxo-2-phenyl-ethyl ester 2-[2-(2-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethyl ester (DMB-HEG-OH) [^{1a}]
(Saha, Ashis K.; Schultz, Peter; Rapoport, Henry, J. Am. Chem. Soc., 1989, Vol. 111, pp.4856-4859; Photolysis occurs in standard laboratory light and product must be kept in complete darkness. Reaction must be done in the dark or under red light (> 630nm wavelength)).

Methyl triflate [²] (Colourless liquid. Handle in glove box) (3.85 g, 2.65 ml, 0.0234) was added dropwise via syringe to a solution of carbonyldiimidazole [³] (White solid. Handle in glove box) (1.9 g, 0.0117 mol) in anhydrous nitromethane [⁴] (Moisture determination done by Coulometric Karl Fischer analysis: 69 ppm H₂O) (15 ml) at room temperature. The mixture was allowed to stir for 15 minutes [⁵] (The solution turned yellow. The reaction is very fast and the 1,1'-carbonylbis(3-methylimidazolium) triflate (quantitative yield assumed) generated is used directly for acyl activation.). A solution of 1,1-carbonylbis(3-methylimidazolium) triflate (prepared as above), was transferred into a suspension of DMB-OH (3.2 g, 0.0117 mol) in anhydrous nitromethane (15 ml) [⁴] (Moisture determination done by Coulometric Karl Fischer analysis: 69 ppm H₂O). After 15 minutes, when CO₂ evolution ceased, a solution of hexaethylene glycol (6.62 g, 0.0234 mol) in anhydrous nitromethane [⁴] (Moisture determination done by Coulometric Karl Fischer analysis: 69 ppm H₂O) (10 ml) was added via syringe. The reaction was quenched with water after 4 hours, and the mixture was extracted into dichloromethane [⁶] (Distilled, not anhydrous, dichloromethane is sufficient. Extraction of the two phases was done using a separatory funnel. The organic phase composed mostly of dichloromethane was recovered as the heavier phase owing to the greater density of the organic solvent to that of water.) (100 ml). The organic phase was washed with 5% aqueous Na₂CO₃ (2 × 50 ml), brine (2 × 50 ml), dried (Na₂SO₄) [⁷] ([Anhydrous] Anhydrous sodium sulphate was used to remove water (2 or 3 spatula scoops)), filtered [⁸] (Gravity filtration using fluted filter paper.) and concentrated in vacuo to yield crude material as a yellow residue. Column chromatography [⁹] (Flash chromatography was used. This involved the application of air pressure onto the solvent above the chromatography media in order to expedite the elution process.) (silica gel, dichloromethane, R_f = 0.05) [¹⁰] (Once all other impurities (which had higher R_f's) were eluted, the eluent was gradually increased to dichloromethane:methanol / 9.5:0.5 to speed up the recovery of the desired product.) yielded 3.41 g (50%) of pure product [¹¹] (Pale yellow oil.). δ_H(200 MHz;

CDCl₃) 7.98-7.93 (2 H, m, aryl), 7.55-7.39 (3 H, m, aryl), 6.66 (1 H, s, CHO), 6.62 (2 H, d, J 2.2, aryl), 6.43 (1 H, t, J 2.2, aryl), 4.34 (2 H, t, J 4.0, OCH₂), 3.78 (6 H, s, CH₃O) and 3.67 (2 H, s, CH₂); m/z (EI) 583 (M⁺, 1%), 298 (57), 255 (50), 149 (65), 105 (94), 89 (100), 77 (45).

Paragraph bridging pages 39 and 40

4.2.1: Preparation of 2-[Bis-(4-methoxy-phenyl)-phenyl-methoxy]-ethanol (DMT-EG) [1]

(Compound is temperature and acid sensitive. Do not heat above 35 °C.)

To a solution of ethylene glycol [2] (Clear liquid. Handle in glove box.) (5.0 g, 0.081 mol) in 15 ml of anhydrous acetone was added triethylamine [3] (Clear liquid. Handle in glove box.) (8.15 g, 11.23 ml, 0.081 mol). After stirring for 10 min, a solution of 4,4-dimethoxytrityl chloride [4] (Orange solid. Handle in glove box. Temperature and acid sensitive reagent.) (13.65 g, 0.040 mol) in 145 ml of anhydrous acetone was added dropwise over a period of 6 h [5] (After complete addition of the 4,4-dimethoxytrityl chloride solution the reaction mixture was orange in colour with the presence of white precipitate (triethylamine salt)). The reaction mixture was then allowed to stir overnight. The resulting mixture was filtered [6] (Under vacuum using a scinter glass funnel. Solution was orange.) and then concentrated *in vacuo*. The resulting oily residue was extracted into dichloromethane [7] (Distilled, not anhydrous, dichloromethane is sufficient. Extraction of the two phases was done using a separatory funnel. The organic phase composed mostly of dichloromethane was recovered as the heavier phase owing to the greater density of the organic solvent to that of water.) (150 ml), washed with water (3 x 75 ml), dried (Na₂SO₄) [8] ([Anhydrous] Anhydrous sodium sulphate was used to remove water (2 or 3 spatula scoops)), filtered [9] (Gravity filtration using fluted filter paper.) and concentrated *in vacuo* to yield crude material as an orange oil. Column chromatography [10] (Flash chromatography was used. This involved the application of air pressure onto the solvent above the chromatography media in order to expedite the elution process.) (silica gel, Dichloromethane:Ether:Et₃N / 96:2:2, R_f = 0.2 yielded 8.1 g (55%) of pure product [11] (Pale yellow oil.). δ_H(200 MHz; CDCl₃) 7.47-7.29 (9 H, m, aryl), 6.86-6.81 (4 H, m, aryl), 3.80 (6 H, s, CH₃O), 3.80-3.73 (2 H, m, CH₂) and 3.26 (2 H, t, J 4.4, CH₂).

Paragraph bridging pages 40 and 41:

4.2.2: Preparation of Diisopropyl-phosphoramidous acid 2-[bis-(4-methoxy-phenyl)-phenyl-methoxy]-ethyl ester methyl ester (DMT-EG-Phosphonamidite) [1] (Compound is temperature and acid sensitive. Do not heat above 35 °C.) To a solution of DMT-EG (2.0 g, 0.0055 mol) in 15 ml of anhydrous dichloromethane [2] (Moisture determination done by Coulometric Karl Fischer analysis: 0.0 ppm H₂O.) was added triethylamine [3] (Clear liquid. Handle in glove box.) (1.39 g, 1.91 ml, 0.0137 mol). After stirring for 15 min, N,N-diisopropylmethylphosphonamidite chloride [4] (Clear liquid. Violently hydrolyses upon exposure to moisture. Use only in glove box.) (1.19 g, 0.006 mol) was added dropwise over a period of 1.5 h [5] (Presence of white precipitate (triethylamine salt) results near the final addition of the chloride.). The reaction mixture was then allowed to stir overnight. The resulting mixture was then concentrated *in vacuo* to give an oily residue. Column chromatography [6] (Flash chromatography was used. This involved the application of air pressure onto the solvent above the chromatography media in order to expedite the elution process.) (silica gel, Ether:Et₃N / 98:2, R_f = 0.8 yielded 2.42 g (84%) of pure product [7] (Hydroscopic white foam/pale yellow oil.). δ_{H} (200 MHz; CDCl₃) 7.52-7.29 (9 H, m, aryl), 6.85-6.76 (4 H, m, aryl), 3.79 (6 H, s, CH₃O-aryl), 3.79-3.61 (4 H, m, CH₂), 3.43 (3 H, d, *J* 12.8, OCH₃), 3.28-3.20 (2 H, m CH) and 1.20 (12 H, d, *J* 7.0, CH₃).

Page 42, second full paragraph:

4.3.4: Photodeprotection of DMB-HEG functionalized substrates.

DMB-HEG functionalized substrates were divided into three batches, two of which were treated to [photodeprotection] photodeprotection prior to solid-phase assembly of oligomers for times of 3 minutes and 1 hour. Photodeprotection was done using a General Electric 85W H85A3 UV mercury lab-arc lamp powered by a MLA-85 Power Supply (Gates, Franklin Park, LI, NY) operated at full power. Substrates were placed in a transparent glass vessel (20 ml volume) to which 10 ml acetonitrile was added. The photodeprotection reaction vessel containing substrates and solvent were rotated at a constant speed (60 rpm) and irradiated at a distance of 60 centimetres relative to the mercury lamp. The photolysis products formed on release of the DMB moiety are shown in the box at the bottom of Figure 5. Following photodeprotection, the

substrates were recovered and washed with 10mL of acetonitrile. The three substrates were kept in darkness and *in-vacuo* until required.

Paragraph bridging pages 47 and 48:

When values of ΔH_{VH} are computed for a given duplex in hybridization buffer at various ionic strengths, values of ΔH_{VH} as a function of temperature are obtained. Recently, Breslauer [[18]] (“Recent Advances in Environmental Chemical Sensors and Biosensors”, ACS Symposium Series, in press) reported that the enthalpy change accompanying denaturation was in fact a function of temperature as a result of a small change in the heat capacity of the system as a result of denaturation, which is contrary to assumptions made hitherto in studies of oligonucleotide hybridization thermodynamics [[26]] (K.J. Breslauer in “Methods in Molecular Biology, Vol. 26: Protocols for Oligonucleotide Conjugates”, S. Agrawal, Ed., p. 347, Humana Press, NJ, 1994). It is therefore possible to use values of ΔH_{VH} obtained at T_m in hybridization buffers of different ionic strengths to compute values of ΔH° at a standard reference temperature, in order to establish a basis of comparison for the relative stability of two different sequences. In general, the enthalpy change for a given process is a function of temperature according to the following relation [[27]] (R.A. Alberty and R.J. Silbey, Physical Chemistry, J. Wiley & Sons, 1st ed., 1992):

Page 49, first full paragraph:

Experiments illustrating the relationship between oligonucleotide immobilization density and the thermodynamic selectivity of nucleic acid hybridizations occurring at solid-liquid interfaces were done using a fibre-optic nucleic acid biosensor based on total internal reflection fluorescence, wherein probe oligonucleotides were covalently bound to the surface of fused silica optical fibres *via* flexible polyether linkers [[28]] (P.A.E. Piunno, J.H. Watterson, C.C. Wust, and U.J. Krull, Anal. Chim. Acta v. 400, p. 73, 1999). Thermal denaturation profiles were obtained for oligonucleotides covalently immobilized to the surface of fused silica optical fibres in an effort to determine if trends observed for hybridization experiments carried out in bulk solution could be extrapolated to describe the behaviour of DNA hybridization at an interface. Since many nucleic acid biosensor schemes involve hybridization of oligonucleotides immobilized to a solid

surface, it is of obvious importance to establish trends in the hybridization thermodynamics for such systems in order to address issues of sensitivity and selectivity.

Paragraph bridging pages 49 and 50:

Studies of hybridization thermodynamics of fully complementary hybrids and those containing a centrally located SBPM were done using dA₂₀-5'-fluorescein and d(A₉GA₁₀)-5'-fluorescein, respectively, at a concentration of 10⁻⁷ M. Thermal denaturation experiments were done using the fibre optic biosensor instrument described elsewhere [[28]] (P.A.E. Piunno, J.H. Watterson, C.C. Wust, and U.J. Krull, Anal. Chim. Acta v. 400, p. 73, 1999). Excitation radiation was delivered to the nucleic acid membrane by means of coupling a beam from an Argon ion laser ($\lambda_{\text{max}} = 488 \text{ nm}$) into the optical fibre. The fluorescent emission was coupled back into the optical fibre and collected at a wavelength of 542 nm. The temperature was ramped in these experiments over the range 25-100°C at a rate of 0.3°C•min⁻¹. Complementary oligonucleotides were introduced in hybridization buffers of various ionic strengths (0.1, 0.3, 0.5 or 1 M NaCl) in an effort to establish the trends in interfacial hybridization thermodynamics as they relate to the ionic strength of the hybridization solution.

Page 51, lines 7-22:

These data illustrate the effect of packing density on the thermodynamics of hybridization. It appeared that the high packing density facilitated some destabilization of the hybridized immobilized oligonucleotides as evidenced by the T_m values which were consistently lower than those observed with the low packing density and medium packing density optical fibre biosensors. Additionally, the sensitivity of T_m to salt concentration in the hybridization buffer appeared to be fairly consistent with observations made in bulk solution, and the three values obtained agree within experimental uncertainty at the 95% confidence level. This supports the notion that there is no significant difference in the ion environments within the nucleic acid membranes brought about as a function of oligonucleotide packing density, as predicted by a theoretical model described elsewhere [[28]] (P.A.E. Piunno, J.H. Watterson, C.C. Wust, and U.J. Krull, Anal. Chim. Acta v. 400, p. 73, 1999). It may be that the differences in T_m observed with the optical fibre biosensor with high oligonucleotide packing density relative to those with the low and medium packing densities is a result of greater interaction between neighbouring

strands, whereby the interactions interfere with the hydrogen bonding between complementary base pairs and reduce the overall stability of the hydrogen bonds. These interactions may also reduce the number of immobilized oligonucleotides that are available for hybridization, similar to what has been reported by Southern [[16]] (E. Marshall, Science, v.268, p.1270, 1995).

Page 54, lines 1-9:

The van't Hoff enthalpy changes at T_m and the standard enthalpy changes corrected to a temperature of 40°C for the different complementary oligonucleotides, oligonucleotide packing densities and hybridization buffer ionic strengths used in these experiments are summarized below in Table 9 and Table 10. Temperature corrections were made as described above according to the method of Breslauer [[18]] ("Recent Advances in Environmental Chemical Sensors and Biosensors", ACS Symposium Series, in press). The reference temperature used for all such corrections was chosen on the basis that it is operational temperature commonly used for hybridization assays conducted in our research group, chosen in order to enhance selectivity and hybridization kinetics.

Pages 54, lines 10-27:

The data shown in Table 9 and Table 10 show that the enthalpic change accompanying denaturation in an interfacial environment is significantly lower than that which is observed in experiments conducted in bulk solution, as shown Table 6. This suggests that there are significant differences in the nature of the hydrogen bonding involved with base pairing in an interfacial environment compared with that which occurs in bulk solution. There did not appear to be a relationship between the packing density of immobilized oligonucleotides and the reduction in the endothermicity of the denaturation. Thus, since observed T_m values are still of comparable magnitude as those which observed in experiments done in bulk solution, it is likely that there is a significant difference in entropy changes accompanying hybridization and denaturation in an interfacial environment, relative to those observed in experiments done in bulk solution. These differences in the entropy changes accompanying hybridization or denaturation may be dependent upon the density of immobilized oligonucleotides, as they may also be affected by the extent of interaction between neighbouring strands. Computation of entropy changes accompanying hybridization or denaturation in an interfacial environment would require

computation of equilibrium constants for the hybridization process, which in turn requires knowledge of the ionic strength within the nucleic acid membrane [[19]] (M. Thompson and L. M. Furtado, Analyst, v. 124, p. 1133, 1999). Similar computations for processes occurring in bulk solution have been known to introduce significant error [[18]] (“Recent Advances in Environmental Chemical Sensors and Biosensors”, ACS Symposium Series, in press), and so these computations for immobilized nucleic acid systems will be left as future work.

Page 56, lines 19-32:

Experiments were done to examine the effects of inclusion of oligomers other than nucleic acids in immobilized films on the selectivity interfacial of nucleic acid hybridization. These experiments were done using a nucleic acid biosensor, based on total internal reflection fluorescence, wherein both probe oligonucleotides and ethylene glycol phosphate (EGp) oligomers were covalently immobilized in two different molar ratios to the surface of fused silica optical fibres via flexible polyether linkers. In these experiments, the method of immobilization used corresponded to that outlined in Example 4. Thermal denaturation profiles were obtained for such nucleic acid films in order to determine if trends with respect to interfacial hybridization using immobilized films comprised of nucleic acid conjugates only were in agreement with those observed in experiments using an immobilized film containing both nucleic acid conjugates and other species not expected to selectively bind nucleic acids. Since it has been shown that the efficiency of hybridization to nucleic acid films is dependent in part on the chemical nature of the interfacial environment [[11, 12, 24]], (T.M. Herne and M.J. Tarlov, J. Am. Chem. Soc., v. 119, p. 8916, 1997; R. Levicky, T.M. Herne, M.J. Tarlov, and S.K. Satija, J. Am. Chem. Soc., v. 120, p. 9787, 1998; U. Maskos and E.M. Southern, Nuc. Acids Res., v. 20, p. 1679, 1992), it is obviously important to examine the effects of film composition on the selectivity of interfacial nucleic acid hybridization.